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Presse Med 52 (2023) 104167

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Update to Quarterly Medical Review -
History of modern pandemics

Gain-of-function and origin of Covid19

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ARTICLE INFO

Article History:
Available online 2 June 2023

SUMMARY

In nature, wild viruses adapted for transmission circulate in many animal species (bats, birds, primates...). Contamination of other animals, including humans, may occur by crossing of the species barrier. Genetic manipulations have been carried out on wild viruses to favor the species jumping and to increase of viral virulence. The aim was to identify the critical genes for pathogenicity. This has been mainly performed on potentially epidemic pathogens, as *Myxovirus influenzae* of avian flu and coronaviruses of SARS and MERS epidemics. These dangerous experiments were subject to a moratorium in the United States (2014–2017). Three years after the emergence of Covid-19, the origin of du SARS-CoV2 remains a mystery. Covid19 appeared in Wuhan, officially in December 2019, but probably during the autumn 2019. The virus was identified in January 2020. It belongs to the genus *Betacoronavirus* (subgenus *Sarbecovirus*). It was at once highly contagious. In addition, the primary isolates were genetically very homogeneous, differing only by two nucleotides without evidence for adaptive mutations. In addition, the Spike protein, a major virulence factor, has a furin site, not found in any other known sarbecovirus. Unlike the SARS and MERS epidemics, no intermediate host has been detected so far. Finally, no other outbreaks were reported at the beginning of the pandemic outside of Wuhan, contrary to what happened with the emergence of SARS (2002) and H7N9 avian influenza (2013). Today, there are two scenarios to explain the emergence of SARS-CoV2. Proponents of the natural origin argue that the bat virus might have directly infected humans, spreading silently at a low level in humans for years, without eliminating the existence of undetected intermediate hosts. This does not explain the origin in Wuhan, far away from the natural virus reservoirs. The furin site would have arisen spontaneously from other coronaviruses. The alternative scenario is that of a laboratory accident after gain-of-function manipulations from a SARS-like virus, or even the occurrence of a human contamination by a natural CoV virus grown on cells in Wuhan.
This article is an update to the Quarterly Medical Review (QMR) devoted to the history of modern pandemics. To access this QMR contents, please go here: https://www.sciencedirect.com/journal/la-presse-medicale/vol/51/issue/3

1. Introduction

The virulence of a virus depends on several factors, including the mode of transmission, tissue tropism, escape from the immune system and survival in the environment. Wild viruses highly adapted to the species in which they circulate recognize specific receptors to enter and multiply in cells. Encountering a new animal species, a virus cannot bind with high affinity to heterologous receptors to invade the cells: this is the main species barrier. Crossing this barrier depends in part on the genetic distance between animal species, the plasticity of viral genomes and the frequency of exposure to the virus. Most often, the first contacts with new viruses fail (epidemiological dead ends), resulting in few sporadic clinical or asymptomatic cases [1–3], as for avian influenza H5N1 in humans. Initial contacts may leave traces in the viral genomes as adaptive mutations, reflecting attempts to cross the species barrier [3]. Serological markers can also be found in contacts. For example, about 80% of living animals sold in 2002 in the Guangzhou markets had antibodies to the virus SARS-CoV1 [4]. For viruses as *Myxoviruses influenzae*, SARS-CoV1 and MERS-CoV, the emergence appears as a sequential process requiring progressive adaptation through intermediate hosts in which mutations occur as well as reassortments in case of co-infections (Fig. 1). Thus, intermediate hosts play a crucial role for crossing the species barrier of these viruses, as for other bat viruses as Hendra virus [5] (Fig. 2).

Advances in molecular biology have provided complete genomes for most pathogens that can be genetically manipulated to identify virulence genes. The virulence of infectious agents can be reduced or enhanced by creating mutations, deletions or insertions in certain genes. These genetic approaches might allow to link the

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https://doi.org/10.1016/j.lpm.2023.104167
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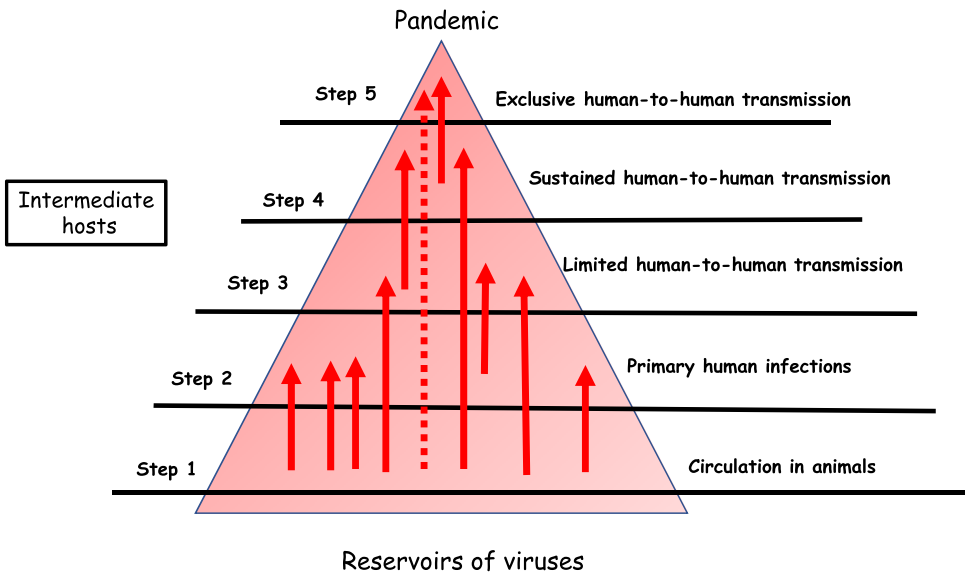

Fig. 1. Crossing the species barrier (adapted from [2].

phenotype to the genotype of pathogenic viruses. There are researchers who claim that understanding viral virulence is of utmost interest to design vaccines and antiviral drugs. However, the *Gain-of-Function* (GoF) manipulations creating hypervirulent viruses are problematic. This is true not only when dealing with emerging *Potentially Pandemic Pathogens* (PPP), such as influenza viruses or coronaviruses, but also with low pathogenic viruses. The US National Science Advisory Board for Biosecurity (NSABB) has

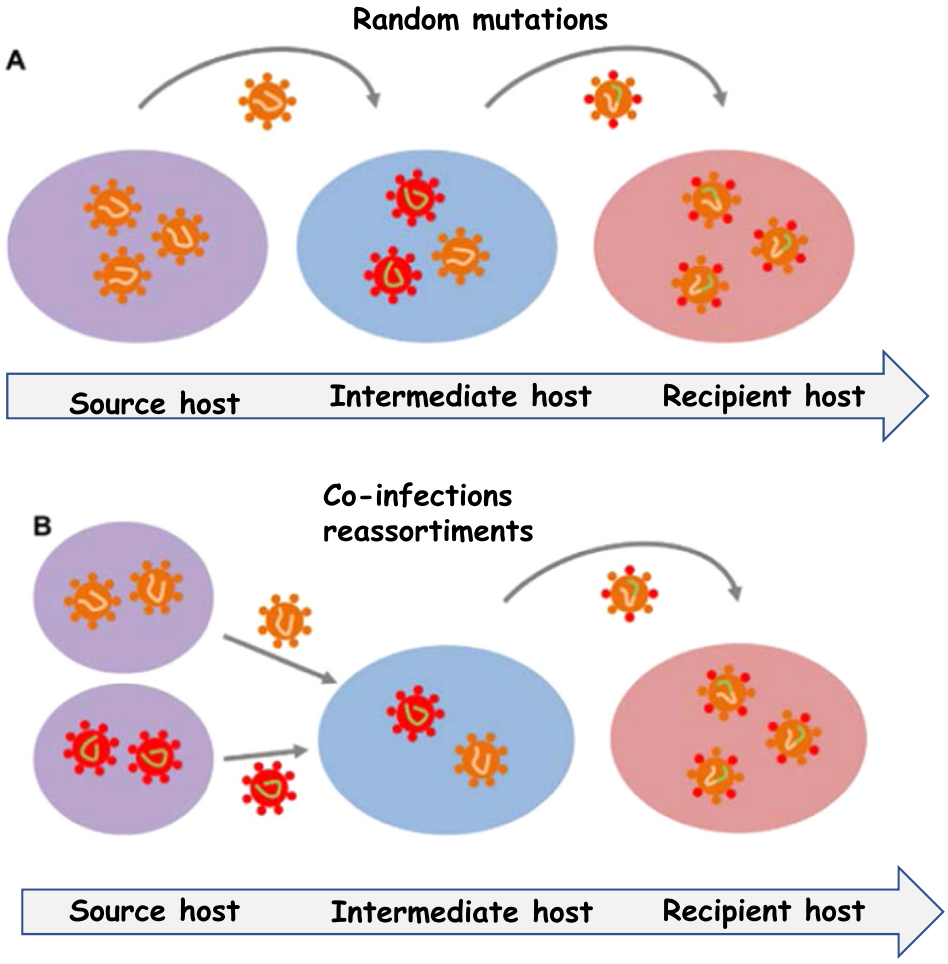

Fig. 2. Gain-of-function (GoF) of a virus in Nature. A. Adaptive mutations through an intermediate host; B. Co-infections of the intermediate host and reassortments.

recently recommended going beyond PPPs, to low pathogenic infectious agents [6]. Indeed, the creation of hypervirulent pathogens poses problems of dual research and biosafety, including risks of escape by laboratory accidents. This is why the GoF are designated *Dual Use Research of Concern* (DURC), emphasizing the potential danger of such experiments [7]. The GoF research must be questioned according to the benefit-risk ratio and must be strictly controlled at several levels, notably before financial funding and before publication of data.

2. Gain-of-function experiments

There are several approaches to exacerbate the viral virulence: [1] random mutations by iterative passages on animals or cell cultures; [2] mutations obtained by directed mutagenesis or reverse genetics (cDNA from RNA viruses is transfected into virus-producing cell cultures to generate mutants by genetic reassortment); [3] random insertion of genetic material in the viral genomes (including DNA shuffling by mixing DNA of different alleles of the same gene). Thus, the cellular tropism of a viral mutant can be altered. It may happen that an experiment leads unexpectedly to a GoF. In 2001, Australian researchers who wanted to develop immunocontraceptive viral vaccines, produced a new, highly virulent mouse poxvirus, the agent of ectromelia (mouse variola). By adding the murine IL-4 gene to the viral genome, the recombinant virus resulted in 100% mortality in all mouse lines, even after vaccination. The novel hypervirulent virus resulted in complete suppression of NK and T responses [8]. Knowing that the Variola virus is closely related to this virus, one may fear that these data become a recipe for a dreadful biological weapon. Was it necessary to publish his results ?

Facing the recent repeated pandemic threats, researchers tried to understand the molecular mechanisms of viral pathogenicity of avian flu (H5N1, H7N9, H7N1), SARS (2002) and MERS (2012), studying virulence factors as the hemagglutinin HA of myxoviruses and the Spike (S) protein of coronaviruses. The aim was to monitor the pool of viruses circulating in wild animal reservoirs and to predict the emergence of new viruses better adapted to humans. This includes GoF experiments on influenza and coronaviruses.

2.1. Influenza viruses

The influenza virus (*M. influenzae*) consists of single-stranded RNA (12−15 kb), encoding 8 genes, including two main virulence genes encoding HA and neuraminidase NA, expressed on the surface of the virus envelope. The genes are carried on separate segments of virus, which facilitates reassortment during co-infections of avian viruses in the pig as intermediate host. Several influenza pandemics over the past century (1918, 1957, 1968, 2009) were the result of such reassortments, involving HA and NA. This is the reason why one fears today the emergence of a new influenza pandemic during avian influenza epizootics. This includes the H5N1 virus reported in Hong Kong in 1996−1997 with a few human cases [9]. This virus is highly contagious and lethal in birds. Sporadically, it can infect humans with a mortality of 60%, but it is not contagious. However, a pandemic alert was triggered in 2005 in Hong Kong, following clustered human cases suggesting human-to-human transmission [10]. Between 2005 and 2011, 562 human cases have been reported, including 329 deaths (60% mortality). It is known that the H5N1 virus recognizes avian sialic acids (SA) (SA-α 2.3 galactose), but not human SA (SA α 2.6 galactose) present on the human upper respiratory tract. In contrast, avian receptors are present in human bronchioles, explaining human cases after deep inhalation of avian virus from close contact with poultry.This explains the high mortality and low viral contagiousness to humans. The question was then to identify in H5N1virus what mutations of HA are required for human SA recognition. In 2012 −2013, three teams in Rotterdam (Netherlands), Madison (USA) and Harbin (China) tried to identify crucial mutations of HA gene, conferring human contagiousness.

Yoshihiro Kawaoka's US team first replaced the HA gene of a human H1N1 virus from the 2009 flu pandemic, with the H5 gene from an avian H5N1 virus [11]. This new virus is not contagious in ferrets, unlike the H1N1 virus. The researchers created random mutations in the H5 gene by reverse genetics and sieved mutants that bind to avian erythrocytes expressing (after treatment) predominantly human SA. They selected 370 mutants in the 120−259 HA region, 9 of which are located in the H5 Receptor Binding Domain (RBD). Four of these mutants inoculated into ferrets via the nasal route were contagious by saliva and aerosols and weakly pathogenic to the ferret. The species barrier between birds and mammals was crossed with only four HA mutations (N186K, S227N, Q226L, G228S) [11]. Ron Fouchier's Dutch team introduced facilitating mutations into a H5N1 virus, two in SA recognition site of HA and one in PB2 gene coding the RNA polymerase active at >40 °C, allowing the H5N1 to replicate at 37 °C in mammals. This adapted mutant was then inoculated into ferrets by nasal route. After 10 successive passages, the resulting mutant viruses induced a fatal disease and became contagious by airborne route between ferrets. The researchers were able to identify 5 critical mutations in H5 (N182K, Q222L, G224S, F627K, N154K), required for mammals adaptation [12,13]. A Chinese team from the Harbin Veterinary Institute also used a similarly adapted H5N1 mutant to infect cells that were transfected with cDNA of the 8 genes of a 2009 H1N1 virus. This resulted in 126 hybrid viruses, 35 of which were found to be highly pathogenic to mice and guinea pigs. These mutants are able to bind to human SA receptors and carry mutations in many viral genes (HA, NA, PB1, PB2, M, NS). They identified HA mutations favoring interactions with the human receptor (G224K, Q226L, G228S) [14]. Thus, these dangerous experiments show that specific mutations in HA gene from H5N1 virus allow attachment to human SA receptors. Interestingly, these experiments indicate that pathogenicity is a complex multi-gene phenomenon, not limited to HA.

In October 2022, a major H5N1 epizootic occurred in mink farms in Spain, affecting nearly 52,000 animals, with an increasing mortality switching from 0.7% initially to 4.3% after three weeks. Genome analysis of contagious mink-adapted H5N1 viruses has detected mutations in PB2 (T171A) polymerase, which would facilitate replication at 37 °C and the genesis of HA mutations [15]. The HA experimental mutations previously described by experimental GoF were not found. These GoF experiments with potentially pandemic viruses led to a moratorium in the USA in October 2014 on financial funding for this type of research, not on banning it. This moratorium was finally lifted in December 2017.

2.2. Coronaviruses

Seven pathogenic coronaviruses are known in humans. Four viruses (designated 229E, NL63, OC43, HKU1) cause mild respiratory infections (rhinitis...), widespread in the infant population. Three are responsible for more severe respiratory infections, as SARS (*Severe Acute Respiratory Syndrome*) in 2002−2003 (8346 cases, 646 deaths, lethality 7.8%.), MERS (*Middle East Respiratory Syndrome*) in 2012 (714 cases, 618 deaths 2012−2015, lethality 35%) in the Middle East with an epidemic in South Korea in 2015 (154 cases, 19 deaths). The MERS pneumonia is not very contagious, but sporadically persists today (> 2000 cases since 2012). Finally, the pandemic Covid19 emerged in December 2019 in Wuhan, evolving in iterative waves [16]. In January 2023, Covid19 has taken more than 17 million deaths in the world according to WHO estimates, and more than 660 million infected cases. Overall lethality would be around 0.6% in Western countries, although it may reach 1−2% in poor countries.

Coronaviruses are enveloped viruses bristled with spicules of S protein, hence its name, Spike. Their viral genomes are single-

stranded RNA of 26–32 kb. The genome of SARS-CoV1 responsible for SARS includes 11 genes encoding the proteins S, E (envelope), M (membrane) and N (nucleocapsid) and an additional gene 1a-1b encoding an RNA polymerase consisting of 16 proteins. After proteolysis, the viral genome encodes 33 functional proteins, all of which play an essential role in the penetration and intracellular replication of virus. SARS-CoV1 and SARS-CoV2 recognize human ACE2 receptors (*Angiotensin-Converting Enzyme 2*). MERS-CoV recognizes the human receptor DPP4 (*Human Dipeptidyl-Peptidase 4*).

In Wuhan, the emergence of SARS in 2002 has stimulated intense research on coronaviruses, including the constitution of a sample collection in Wuhan from bats captured in caves of South China and Southeast Asia, from 2004. A stock of nearly 15,000 samples (blood, saliva, urine, etc.) has been built up, allowing identification by RT-PCR and sequencing of about 220 SARS-like coronaviruses. About 100 sequences have been published.

It is well established that bats constitute the wild reservoir of the three pathogenic human coronaviruses. The SARS-CoV1 virus reservoir was identified in 2005 in bats (*Rhinolophus spp*) by *Wuhan Institute of Virology* (WIV) teams [17]. In a cave in Yunnan province in 2017, these researchers discovered 8 unknown bat viruses closely related to SARS-CoV1, including two with a functional protein S and 6 with deletions of RBD of protein S. The S protein of virus Rs4874 was identical (99.9%) to that of SARS-CoV1 [18]. Intermediate hosts carrying viruses very similar to SARS-CoV1 were also identified in the webbed civets, raccoon dogs and badgers. Similarly, the intermediate host of MERS-CoV has been shown to be the dromedary contaminated by bats.

In 2008, a GoF experiment was performed by Ralph Baric's US team at Chapel Hill (North Carolina) on a wild strain of SARS-like coronavirus (Bat-ScoV) that poorly penetrates human cells expressing ACE2. From the published nucleotide sequence, a cDNA was synthesized and transfected into cultured cells, thereby producing a replicative virus. By replacing the Spike-RBD with that of SARS-CoV1, the virus very easily invades cell cultures and becomes pathogenic in mice. This demonstrates the importance of the RBD of Spike [19].

In 2013, Chinese researchers discovered several coronaviruses (RsSHC014, Rs3367, SL-CoV-WIV1) in bat samples from the province of Yunnan, whose S sequences are very similar to that of SARS-CoV1. These viruses can bind to human, civet and *Rhinolophus* ACE-2 receptors expressed by HeLa cells where they can multiply [20]. Phylogenetic studies showed significant differences in 14 aminoacid residues, which favored Spike binding to these receptors, including 5 critical substitutions for the host spectrum (Y442, L472, N479, D487, Y411).

During the period of the US embargo (2014–2017), US and Chinese teams of Ralph Baric and Zengli-Li Shi, cooperatively performed GoF experiments in 2015 using the "skeleton" coronavirus M15. This is an avirulent SARS-CoV1 virus, adapted to humanized transgenic mice expressing the human ACE2 receptor. This M15 virus is unable to penetrate human cells. The addition to M15 of gene S from a wild-type SARS-like coronavirus (SHCO14-CoV) found in *R. affinis* generated a new virus capable to recognize multiple orthologs of human ACE-2 (Fig. 3). This recombinant virus was serially transmitted in vitro to primary human respiratory epithelial cells, where it reached high titers. Passage in humanized mice showed extensive replication in lungs. This mutant was no longer neutralized by anti-SARS-CoV1 antibodies neither protected by SARS vaccines [21].

In 2014 during the moratorium, a US team from the University of Iowa implemented GoF experiments on MERS-CoV, which bears two furin sites in protein S [22]. The wild-type virus was propagated in transgenic mice expressing the human DPP4 receptor for this virus. Nasal infection of these mice does not result in disease, but after 30 passages the virus causes a lethal infection with a 100-fold increase of growth in lungs, as compared to the parental virus. Genetic analysis of these strains detects 13 to 22 mutations, several affecting the MERS protein S [23]. These mutations make the virus sensitive to cellular proteases. Very recently, in October 2022, a team from the

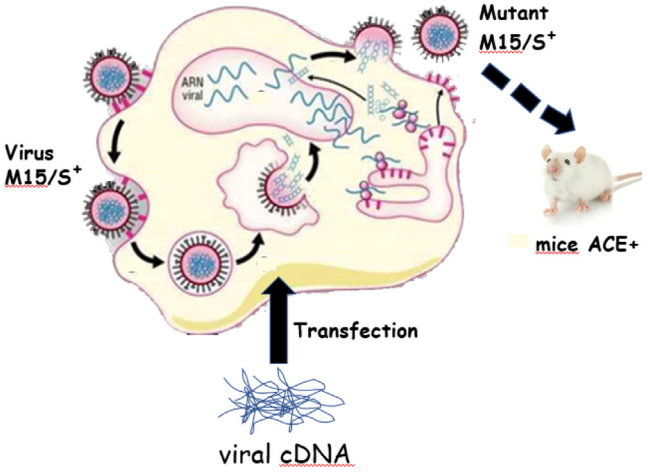

Fig. 3. Example of GoF experiment: creation of chimeric coronavirus: cells infected by M15 virus (avirulent mutant SARS-CoV1) were transfected with cDNA from a wild-type bat coronavirus isolate (SHCO14-CoV). Chimeric mutants are produced in culture and used to infect transgenic mice expressing human ACE2. After serial passages, mutants become highly pathogenic [21].

Boston School of Medicine constructed from the original Wuhan-Hu-2 virus, a recombinant virus carrying the S protein of an omicron BA.1 variant, known to be more contagious and less virulent than the original virus. This variant carrying numerous mutations in the Spike-RBD escaped humoral vaccine-induced immunity. While the omega virus causes a mild non-lethal infection in humanized K18-hACE mice, the chimeric virus carrying the S gene from omicron virus induces an 80% fatal infection [24].

3. Emergence of SARS-CoV2

According to Chinese authorities, the first case of severe pneumonia occurred on December 8, 2019, in Wuhan (population 11 million) in Hubei Province [25]. The new virus was quickly identified [26,27]. It is immediately recorded that 33% of initial cases defined by pulmonary symptomatology were associated with a downtown fish market (Huanan Seafood Market) [28]. As of February 5, nearly 50% of the first 99 reported cases were reported to have attended this market [29], where many living animals were sold, kept in unsanitary conditions and possibly shedding viruses [30]. This market was closed on January 1, 2020.

Events then followed one another very quickly. Containment of the city was declared on 23 January 2020, a few days after the Lunar New Year celebrations on 20 January, but nearly 5 million people have left Wuhan to join their families throughout the country, thus dispersing the virus. The first hypothesis was that infection came from live animals sold in the market, a scenario similar to that of SARS in November 2002 in Foshan, near Guangzhou, in the Guangdong province. Chinese authorities have said that they have not detected virus in living animals at the Huanan Seafood Market, nor in the many factory farms that breed minks, foxes and raccoon dogs. Only some environmental samples from the Huanan market were found to contain the virus found by RT-PCR [25]. Therein begins the enigma of the Covid-19 origin and the questions that arise. A recent US Senate report in October 2022 revived the claim that SARS-CoV2 escaped from a high-security laboratory in Wuhan [31].

3.1. Epidemiology of Covid-19

The occurrence of natural zoonotic infections is determined by the degree of exposure to viruses carried by an intermediate host. The overpopulation of live animals sold in markets, often in poor sanitary conditions, implies close promiscuity favorable to viral spread

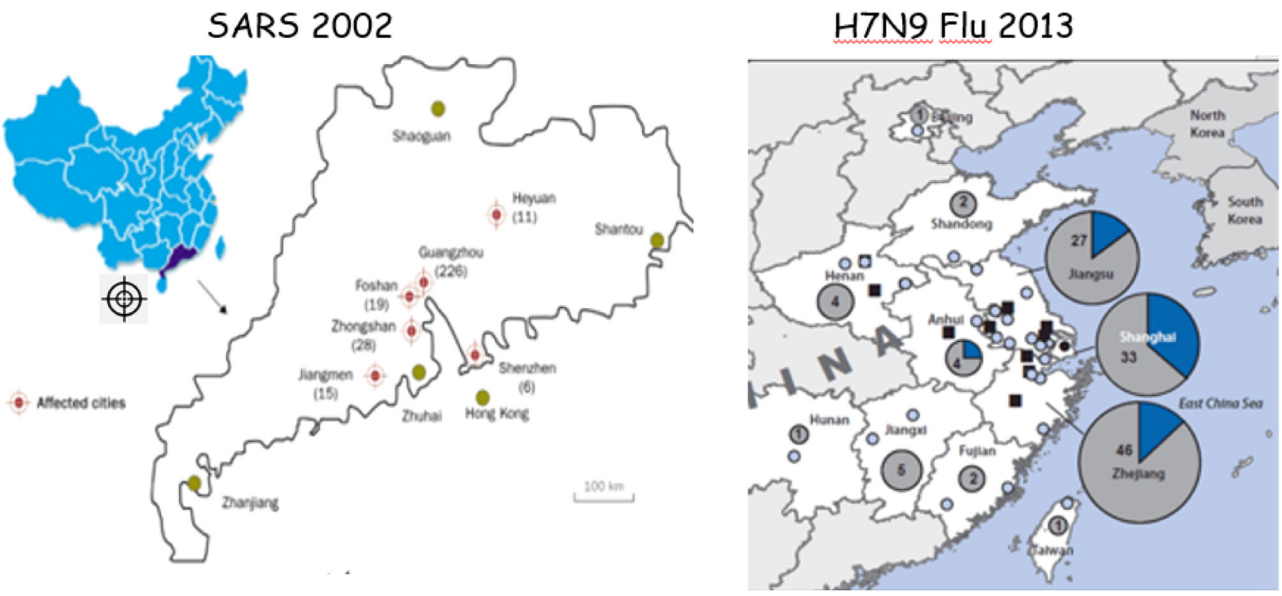

Fig. 4. Epidemic outbreaks at the start of SARS and H7N9 influenza epidemic. A. Outbreaks of SARS in Guangdong province, according to [32]. B. H7N9 avian influenza outbreaks in the Shanghai area, from [34].

between animals and humans, as well as during the transit across the country to markets. Therefore, one might expect that many limited human clusters would occur during the emergence throughout various places during transportation. The Chinese epidemiological surveillance network did not report any pneumonia outbreaks outside Wuhan in the last quarter of 2019 [25]. For the SARS emergence in 2002, there were at least five human clusters followed by epidemic dissemination of virus. Multiple geographically distant locations were observed in the markets of Foshan, Guangzhou, and Hong Kong, where the virus first emerged [32,33] (Fig. 4A). Intermediate hosts, such as the webbed civet and raccoon dog, were identified in the early months of SARS epidemic. The other example is the H7N9 avian influenza virus that occurred in humans in 2013 in China. This limited human epidemic began with multiple independent viral introductions into humans in multiple locations, although the total number of human cases was less than 500 [34] (Fig. 4B). Furthermore, the intermediate host of SARS-CoV2 could not be identified, despite more than 80,000 specimens from a wide range of animal species in China (Fig. 5). The only example of animal-to-human transmission of SARS-CoV2 was reported in factory farms in Denmark where the virus has been transmitted from minks to animal house staff [35].

Very recently, a Chinese team published the results of the virological survey performed at the *Huanan Fish Market* after its closure on January 1, 2020. Out of 457 samples collected from 18 animal species (118 individuals) and identified by RT-PCR and direct sequencing, SARS-CoV2 RNA was not found in any of these samples. In contrast, of 718 environmental samples, 40 were positive by RT-PCR between January 1 and March 2, 2020 [36]. The metagenomic DNA sequences of environmental samples were posted on the Gisaid database. A French researcher has identified mitochondrial DNA from various animals in virus-posive samples, including raccoon dog and civet, animals not present in the market according to Chinese authorities. These species are known to be capable of spreading SARS-CoV-like coronaviruses [37]. These results might be in favor of an undeclared intermediate host, but do not establish a causal link, due to natural DNA persistence in the environment.

3.2. *The singularities of SARS-CoV2*

SARS-CoV2 was identified in early January 2020. It is an enveloped single-stranded RNA virus of 29,600 nucleotides. Classified as a β-coronavirus, it belongs to the *sarbecovirus* subgenus (*SARS-like β-coronavirus*). Analysis of SARS-CoV2 genome shows three important features: [1] a very low diversity among initial isolates; [2] a strong affinity of these isolates for the human ACE2 receptor; [3] the presence of a furin site absent in other known sarbecoviruses [38,39]. The first identified viruses differed by only two nucleotides: Wuhan-Hu-1 (clade B) and Wuhan/ME-WHO/2019 (clade A), suggesting a very recent origin. This is in contrast with the very rapid diversification of virus by mutations subsequently observed, ultimately resulting in iterative waves of α, β, γ, o mutants during the next three years (Fig. 6). This recent origin is also predicted by epidemiological

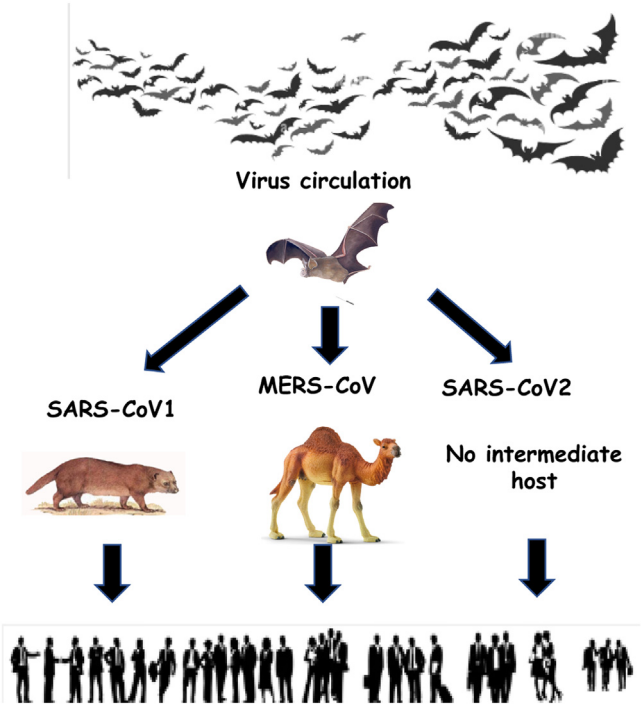

Fig. 5. Circulation of coronaviruses in *Rhinolophus spp* bats, and crossing of the species barrier by intermediate hosts for SARS-CoV1 (civet), for MERS-CoV (dromedary). So far, no intermediate hosts have been discovered for SARS-CoV2.

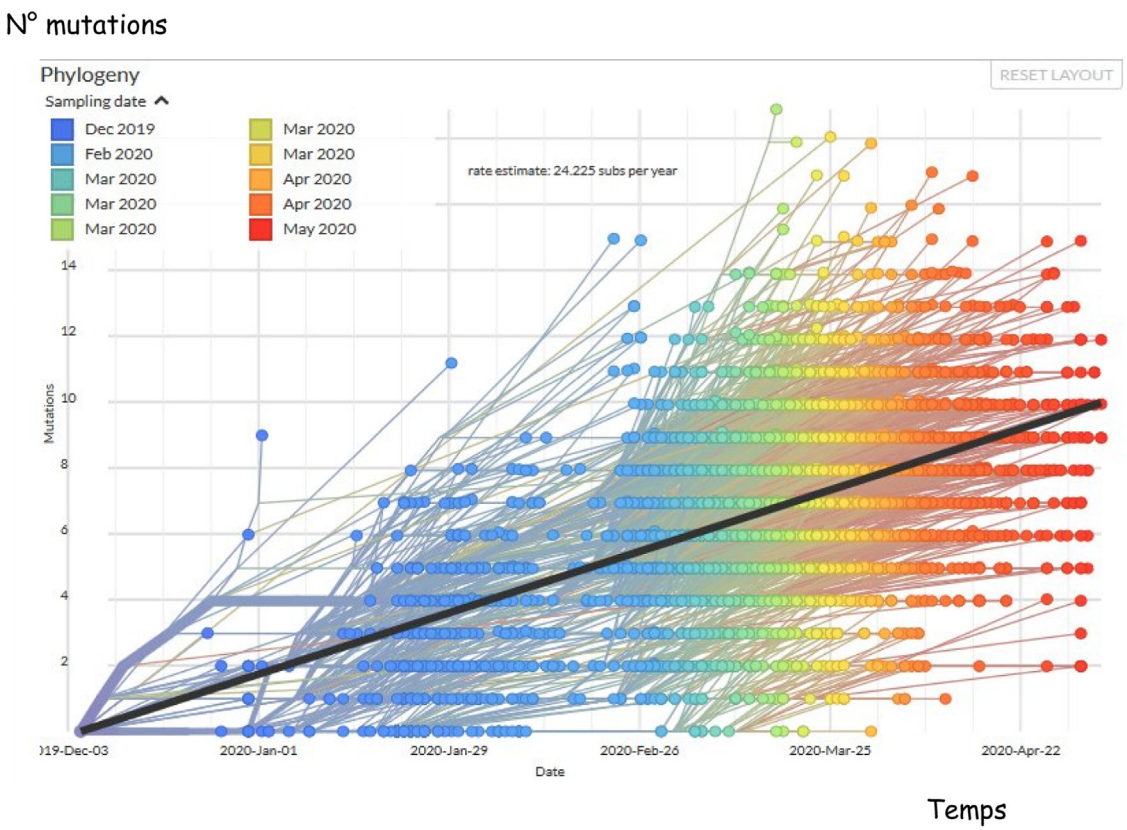

Fig. 6. Evolution of SARS-CoV2 mutations from December 2019 to April 2020. It is shown the initial homogeneity of virus that rapidly diversifies with multiple mutations. Gisaid https://nextstrain.org/ncov/global/2020−05−14.

modeling between mid-October and mid-November 2019. Surprisingly, the emerging viruses were at once highly adapted to human-to-human transmission, i.e., they recognized human ACE2 with a high affinity. It is known that wild bat viruses similar to SARS-CoV1 are often unable to infect human cells because these viruses are adapted to the bat ACE-2 [40]. Moreover, adaptive mutations reflecting prior virus circulation in the hosts are not detected in the SARS-CoV2 genome, in contrast to observations made on SARS-CoV1 in 2002.

The discovery of a furin site in SARS-CoV2 has been much discussed. Furin is a ubiquitous intracellar protease localized to the Golgi apparatus and the nucleoplasm, cleaving the S protein and other proteins from various other viruses. The furin site consists of four amino acids (pro-arg-arg-ala/PRRA), corresponding to an insert of 12 nucleotides (T-CCT-CGG-CGG-GC[A]). It is located at the junction of the two subunits S1 and S2 of S protein (Fig. 7). The cleavage promotes the penetration of virus into cells. Furin sites are present in some β-coronaviruses (other than sarbecoviruses), notably in MERS-CoV which presents two furin sites [23], and also in avian influenza viruses and Ebola virus. No furin sites can be found in the other known sarbecovirus sequences.

3.3. Coronavirus research in Wuhan

Since the deadly SARS epidemic of 2002−2003 and the pandemic threat of H5N1 avian flu in 2005, Chinese authorities have focused on viruses potentially dangerous viruses. For example, in late 2018 −2019, a deadly African swine fever epizootic due to a DNA Asfivirus spread throughout China and Southeast Asia, resulting in an economic disaster. The aim of the research was to prevent the emergence of unknown pathogens, to develop vaccines and medical countermeasures. Wuhan has thus become a center of excellence for virus research. Teams from the WIV and Wuhan Chinese CDC were sent to collect samples on the field in South China and Southeast Asia, where bats abound, more than 1600 km from Wuhan (Fig. 8). It is accepted that the SARS-CoV2 originates from *Rhinolophus spp*, living in South China and Southeast Asia, but absent in Wuhan. Among the coronavirus isolates most closely related to SARS-CoV2 is RaTG13 (96.1% nucleotide similarity) collected in 2013. The previous year in April 2012, an outbreak occurred in an abandoned copper mine of Tongguan near Mojiang (Yunnan), where miners were cleaning from bat guano. Six workers between 30 and 63 years of age developed severe acute pneumonia and were hospitalized in the provincial capital of Kunming. Three died [41,42]. The Chinese authorities indicated that the patients' samples sent to Wuhan did not reveal the presence of coronavirus. They specified that pneumonia was caused by a fungus [25]. The sequence of RaTG13 was finally published in February 2020 [43]. A French team directed by Marc Eloit has collected in Laos several β-coronaviruses very close to SARS-CoV2 from bats, including BANAL-52 (96.8% nucleotide similarity), BANAL-103 and BANAL-236 [44]. Phylogenetic studies show that all these wild-type viruses have diverged for decades (Fig. 9).

In Wuhan, there are eight institutions working on coronaviruses in 2019 with microbiological safety laboratories (BSL2−3−4). This includes the Wuhan Institute of Virology (WIV) located in two places, one at the historic Xiaohongshan site in the heart of the city, and another at the Zhengdian site about 20 km away in the southern suburbs for the BSL-4 laboratory recently opened in 2018. The virus collection is stored next to the BSL-4 laboratory. Security infrastructure in most other institutions, including BSL-3 and animal facilities, were recently established in the last five years. These include Wuhan Chinese CDC, Hubei Chinese CDC, Hubei Animal CDC, Wuhan Institute of Biological Products harboring the vaccine production branch of Sinopharm, Wuhan University, Huazhong Agricultural University and

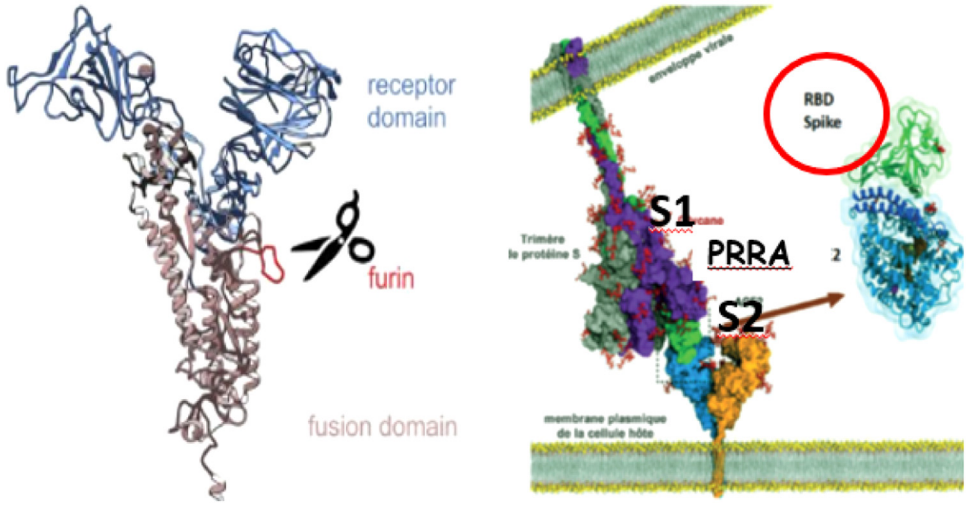

Fig. 7. Schematic of action of furin: it is a cellular protease that cleaves the S protein between subunits 1 and 2, recognizing a specific PRRA site. This facilitates the virus entry in cells after interaction with ACE2.

Wuhan Entry Exit Inspection. Thus, coronaviruses are handled at 9 different sites, including numerous BSL2 laboratories, six BSL3 laboratories, three ABSL-3 animal facilities, and one BSL4 laboratory (WIV). The Wuhan Chinese CDC is located close to the Huanan Seafood Market.

The WIV has conducted genetic manipulations and chimera construction experiments in BSL-2–3 laboratories. The researchers used humanized transgenic mice expressing human ACE2 receptors. This allows them to create and selected adaptive mutations by iterative passages in mice and to evaluate the efficacy of vaccines. They also test the virulence of coronaviruses on civets which have an ACE2 close to the human receptor [45]. The most dangerous experiments are those done in animal houses that produce highly infectious aerosols difficult to detect. A Chinese biosafety expert recommended in May 2019 that all experiments in animals should now be done in BSL3 or BSL 4, no more in BSL2 [46]. It must be added that field collection conditions were often not safe in terms of protective equipment and bat handling.

In March 2018, a joint funding application was submitted to DARPA (*Defense Advanced Research Projects Agency*), a U.S. research funding agency. It involved teams from WIV and the U.S. non-governmental organization *EcoHealth Alliance*. It is proposed to search bat coronavirus samples collected in Yunnan by the WIV team, which are genetically close SARS-CoV1 and possibly bearing furin cleavage sites. The project stipulates that, in case of failure to find such viruses, researchers intend to manipulate SARS-like coronaviruses to increase binding affinity to human lung tissue and possibly to insert furin sites at the same location as those found in SARS-CoV2 [47]. This project was rejected by DARPA. Addition of furin site has been made in the past in various viruses. For example, researchers at Huazhong Agricultural University in Wuhan in 2015 inserted a furin site into an α-coronavirus responsible for Porcine Epidemic Diarrhea, facilitating entry and replication in cell culture [48]. Similarly in 2019, researchers in Beijing modified a furin site in the coronavirus of the Infectious Poultry Bronchitis, with increased virulence and neurotropism of virus [49].

4. The two scenarios for the origin of Covid-19

The first scenario is that of a natural origin of SARS-CoV-2 [50–52]. The virus would have directly contaminated humans from bats, with a silent, low-level infections not detected by China's epidemiological surveillance system. The alternative is that there was an intermediate host that has not yet been detected. During the adaptation phase, the virus would have naturally acquired the furin site by accumulation of mutations, implying that random has allowed the juxtaposition of 4 amino-acids at the right place at the S1/S2 junction. It is also possible to hypothesize recombination with other coronaviruses carrying furin sites. Scientists holding up this scenario support

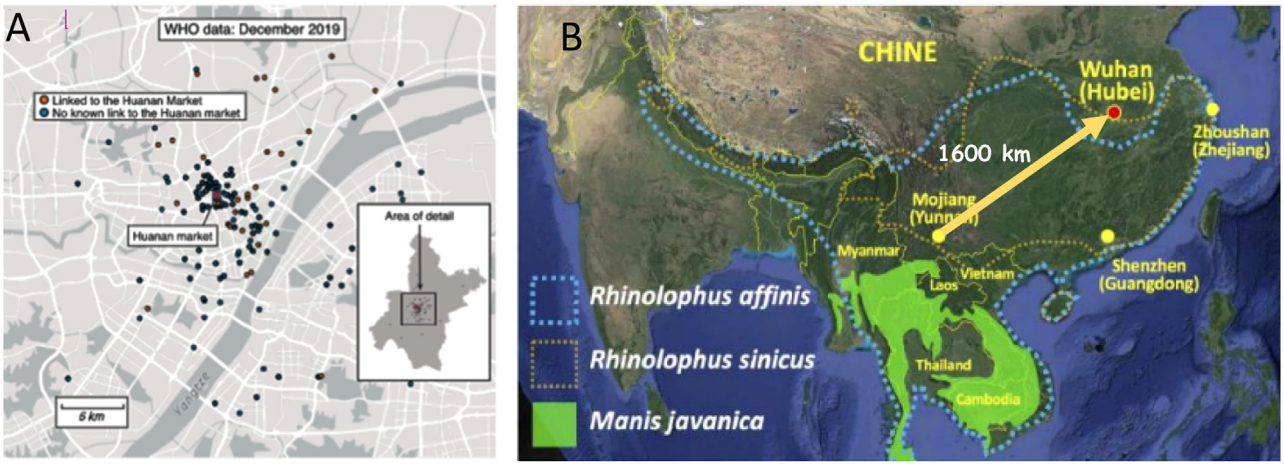

Fig. 8. Origin of SARS-CoV2. A. The first cases that appeared at Huanan Seafood Market in Wuhan (December 2016) from [30]; B. Geographical areas of *Rhinolophus spp* bats, reservoirs of coronavirus, 1600 km from Wuhan.

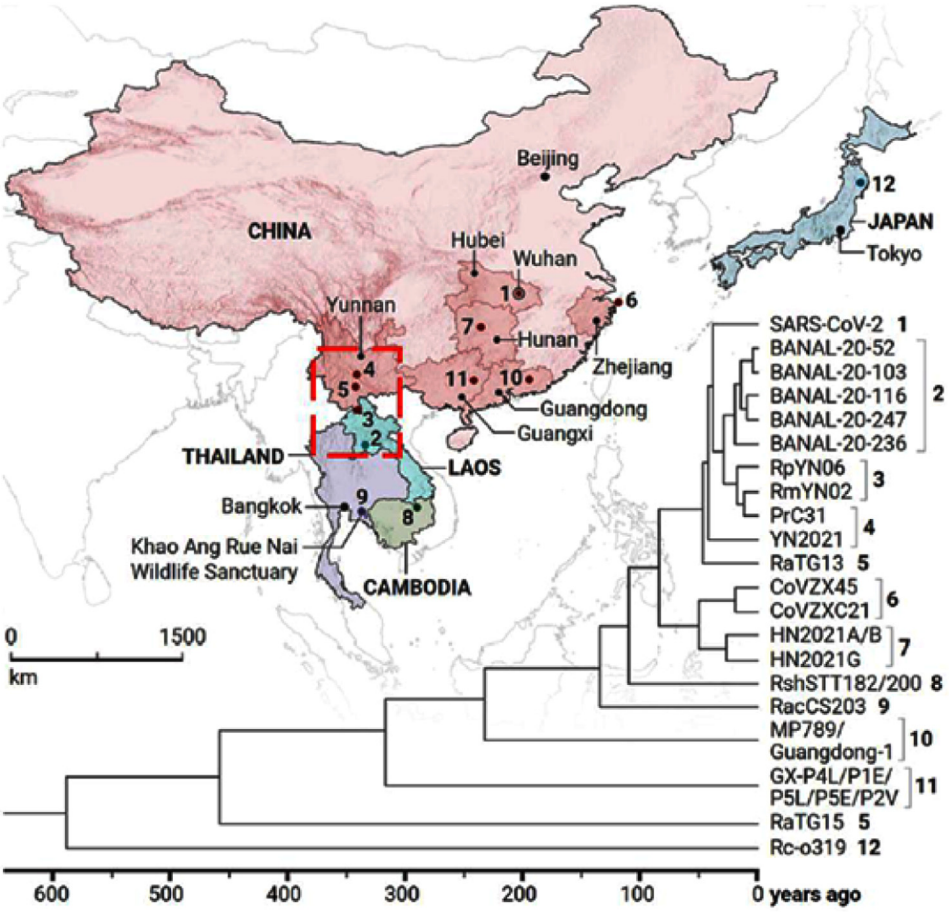

Fig. 9. Phylogenetic studies showing the areas where the most closely related virus to SARS-CoV2 were isolated. Adapted from [44].

the idea that a predecessor of SARS-CoV2 might have circulated silently in human populations for years until acquiring the cleavage site, then triggering the Covid19 pandemic.

The alternative scenario is that of a laboratory accident after gain-of-function manipulation of SARS-CoV2. First, there is the absence of identified intermediate hosts after three years of pandemics. Second, why Wuhan? This megapolis where the first cases of Covid-19 were detected is remote from the areas of bat reservoirs. In the early phase of the pandemic, the absence of secondary outbreaks that would have accompanied the trade of living animals is surprising. During the emergence of other recent viral respiratory diseases transmitted by animals on markets, as SARS and H7N9 avian influenza, multiple scattered clusters were observed [32–34]. In Wuhan and elsewhere, researchers have practiced GoF on sarbecoviruses. According to publications, chimeric viruses were created in 2015, followed by 8 more viruses in 2017, two of which were pathogenic to humanized mice. All indications are that the origin of SARS-CoV2 in December 2019 was very recent, a hypothesis corroborated by epidemiological models. We also observe very low genetic diversity of initial isolates, contrasting with the high diversity that viruses can deploy in a few weeks. Moreover, the SARS-CoV2 was immediately highly contagious, witnessing a remarkable adaptation of this bat virus to humans. The presence of a furin site in SARS-CoV2, which is not found in any other known sarbecovirus, is also a singular feature that remains to be explained. Moreover, the hypothesis of a researcher be infected by a non-engineered natural CoV grown on cells in Wuhan cannot be eliminated.

Accidents may occur even in high security microbiology laboratories (BSL3–4). For example for the 2002 SARS-CoV1, four lab leaks (1 in Singapore, 1 in Taïwan and 2 in Beijing) were reported after manipulations in such laboratories [53–56]. Similarly, the reemergence in 1977 of a H1N1 epidemic virus ("Russian flu"), which was eradicated during the 1957 asiatic pandemic, was a laboratory accident, most probably linked to a vaccine trial in the Soviet Union or China [57–59]. This results in a flu pandemic (1977–1979), then the H1N1 virus persists until the porcine pandemic in 2009. One must also remember the three accidents linked to the handling of smallpox virus in high-security laboratories that occurred in 1972 in London and in 1966 and 1978 in Birmingham, resulting in 80 smallpox cases and three deaths [60]. For respiratory viruses, as coronaviruses, influenza viruses or smallpox, the most dangerous manipulations are those that take place in animal houses and cellular cultures, producing infectious aerosols difficult to detect. We must also mention the risks during field collections, requiring protective equipment and handling of bats. Breaches in the security systems of the WIV have also been suspected, particularly in mid-November 2019 [36]. The natural or accidental origin of SARS-CoV2 remains an unsolved conundrum. But sooner or later, the truth will emerge.

In conclusion, the GoF experiments on viruses require strict controls, including an assessment of the benefit-risk and of a possible dual research, and the appropriateness of publishing. Biosafety and biosecurity conditions in laboratories and animal facilities must be at the forefront. Finally, it is important that the scientific community must adhere to this approach through educational actions.

Declaration of Competing Interest

The author has no conflict of interest and no financial support.

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